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APPLICATION TECHNOLOGY HEALTH EFFECTS PROGRAM PROGRESS REPORT JANUARY 1986–DECEMBER 1986

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Rockwell International

North American Space Operations
Rocky Flats Plant
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APPLICATION TECHNOLOGY HEALTH EFFECTS PROGRAM PROGRESS REPORT JANUARY 1986–DECEMBER 1986

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L. C. Smith

BERYLLIUM ANTIBODY DETECTION METHOD

S. M. Clarke and C. W. Barrick

OBJECTIVE

The objective of this project was to develop a method to detect antibodies specific to beryllium.

PRIOR WORK

Previously, we reported work to design and develop a method for detecting antibodies specific to beryllium.[1] Currently, we are optimizing the parameters of this method. A serious limitation has been that we are unable to locate a continuing source (donor) of blood that is "high" in beryllium anitbodies.

METHOD

The method we developed was a modification of an enzyme linked immunosorbent assay (ELISA) where we bound the antigen (beryllium) to 96 sticks located on F.A.S.T.™ plates and exposed the antigen to antibody present in individual plasma. The antigen/antibody complexes were located using a biotin-avidin amplification system.

EXPERIMENTS AND RESULTS

Different ways of attaching the antigen (beryllium) to the plates were tried. When we used the standard format of drying beryllium ($BeNO_3$) in the bottom of wells located in individual plates, with and without adhesives, we were unable to get the antigen to attach. The Research and Development Department of Becton Dickinson Laboratory sent us negatively charged F.A.S.T. plates to test. These plates were charged for increasing periods of time. All plates were tested, but only the two most highly charged plates were successful in binding the antigen. A third method that we tested was to coat the entire plate with beryllium (as metal) using vacuum evaporation techniques of the Rocky Flats Coating Lab. This method was also successful. We are currently comparing results obtained using the negatively charged plate and $BeNO_3$ with the vacuum beryllium metal coated plate.

We tested the response of the assay when we attached various amounts of antigen (beryllium) to F.A.S.T. plates. We found that the greater the amount of beryllium exposed to the plates, the greater the color response obtained. This response indicated that we needed to saturate the system with antigen to obtain optimum results.

Using the negatively charged F.A.S.T. plates, we checked the pH of the beryllium solution which allowed for best attachment. At 0.46 pH we assumed that beryllium was in its ionic form and at 4.89 pH we assumed that some colloids were present. Best results were obtained using beryllium at 0.46 pH. When the plate contacted plasma, the pH rose and probably formed colloidal beryllium hydroxides or oxides. We tested two reagents (moducyte and normal goat serum) to block nonspecific binding sites. The same results were obtained with both reagents. More work is being done to reduce background interference.

By using the F.A.S.T. system, we limited ourselves to Falcon 96™ well plates. We found that Probind plates and the plates provided with the F.A.S.T. system were satisfactory, but Falcon 96 well plates #3070 and #3072 could not be used due to the great variability recorded when the plates were read empty.

The possibility of nonspecificity and/or cross reactivity was tested by attaching magnesium and calcium to the negatively charged F.A.S.T. plates.

These two elements are chemically similar to beryllium and we felt it was necessary to check for any cross reaction to them. Both tested negative. If we can get magnesium and calcium coated plates made, we will test for cross reactivity using this technique also.

The specificity of the reagents in the biotin-avidin system was examined by running the assay and leaving out one reagent at a time. The assay was negative each time showing that there was no nonspecific sticking of any reagent, and that each reagent was important for the assay to function. A report by Fischer and Krampitz[2] states that beryllium binds to some proteins and peptides found in egg white. Avidin is an egg white protein, but we found no nonspecific adherence of beryllium to avidin in our assay.

To date, we have tested 29 individuals with no known contact with beryllium, one individual on steroid treatment but no beryllium disease, one individual diagnosed with sarcoidosis, five individuals with cases of diagnosed chronic beryllium disease and one individual whose condition is suspected beryllium disease. The results are shown in Table 1.

Plasma from the participating test individuals was diluted 1:2 in Dulbecco's phosphate buffered

TABLE 1. Beryllium Antibody Screening Assay

Diagnosis	Test Rdg (OD Units X 10^3)	Control Rdg (OD Units X 10^3)	Difference (Test Reading-Control)
1. No Known Be Disease	9	36	-27
2. No Known Be Disease	4	115	-111
3. No Known Be Disease	24	58	-34
4. No Known Be Disease	45	84	-39
5. No Known Be Disease	47	94	-47
6. No Known Be Disease	38	120	-82
7. No Known Be Disease	18	104	-86
8. No Known Be Disease	12	32	-20
9. No Known Be Disease	104	155	-51
10. No Known Be Disease	7	54	-47
11. No Known Be Disease	33	84	-51
12. No Known Be Disease	64	158	-94
13. No Known Be Disease	42	82	-40
14. No Known Be Disease (on Steroid R_x)	-11	76	-87
15. No Known Be Disease	175	235	-60
16. No Known Be Disease	238	168	70
17. No Known Be Disease	399	209	190
18. No Known Be Disease	212	160	52
19. No Known Be Disease	158	201	-43
20. No Known Be Disease	142	206	-64
21. No Known Be Disease	144	250	-106
22. No Known Be Disease	206	248	-42
23. No Known Be Disease	195	193	2
24. No Known Be Disease	269	220	49
25. No Known Be Disease	516	419	97
26. No Known Be Disease	388	180	208
27. No Known Be Disease	442	478	-36
28. No Known Be Disease	40	56	-16
29. No Known Be Disease	272	340	-68
30. Sarcoidosis	154	145	9
31. Chronic Be Disease	380	254	126
32. Chronic Be Disease	318	270	48
33. Chronic Be Disease	126	228	-102
34. Chronic Be Disease	317	280	37
35. Chronic Be Disease	475	525	-50
36. Chronic Be Disease Suspect	672	34	638

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saline. Duplicate samples were run and averaged. The test readings were obtained from assays using beryllium attached to F.A.S.T. sticks. The control readings were obtained from assays run identical to the test assays except that there was no beryllium present. The difference reading in Table 1, last column, indicates the amount of beryllium antibody present.

Most of the individuals who had no known contact with beryllium showed low optical density (OD) readings when the control readings were subtracted from the test readings. However, two samples showed significantly higher difference readings (190, 208). In Table 1, refer to numbers 17 and 26. The individual with a sample showing the 208 reading was resampled two months later and the reading decreased to -36. See Table 1 number 27. A check with Industrial Hygiene revealed that he could have been exposed to beryllium (just below the action level) approximately two weeks before the first test. The first individual was not retested.

Of the five diagnosed chronic beryllium disease patients that we tested, only one (difference reading of 126) has been confirmed by lymphocyte transformation assays, lung biopsy and x-ray patterns. The chronic beryllium disease suspect, who gave us our highest reading, has been confirmed as having chronic beryllium disease. We have also used the assay to test five lung lavage fluid samples. These results were hard to interpret since there are few B cells in the lungs, and we had no information to relate lung fluid volume to lavage volume. These specimens, as well as some of the blood samples, were sent to us from National Jewish Hospital, Denver, CO. One blood sample was from Los Alamos National Labortory.

FUTURE WORK

The legal department has drafted an informed consent form for employees to read and sign when their blood is tested. The medical department is forming a Human Services Committee to review experiments performed on employees.

We are investigating better ways of measuring, calculating and reporting our data to replace the OD difference units. We have also initiated an employee screening experiment to detect high titers. These samples will be tested for total IgG, IgG subclasses, and IGE. If a positive titer sample can be found, then experiments testing our assay for reproducibility and specificity will be done. Inhibition studies will be included.

If the assay can be shown to be reproducible and specific, it will be used to test for antibodies against other metals. Other uses for the assay are for screening beryllium workers, for diagnosis, and for studying individual immune responses.

REFERNCES

1. L. C. Smith, *HS&E Application Technology Semiannual Progress Report July Through December 1985,* RFP-4036, Rockwell International, Rocky Flats Plant, Golden, CO, May 1, 1987.

2. Von Petra Fischer and G. Krampitz, "Mechanism of Biochemical Action of Beryl Binding in Egg White," in *Zeitschrift fuer Tierphysologie, Tierernaehrung und Futtermittelkunde, 50 (3),* pp 125-131, 1983.

CHARACTERIZATION OF THE *IN VITRO* HUMAN ERYTHROCYTE ACUTE TOXICITY ASSAY

S. M. Clarke and C. W. Barrick

OBJECTIVE

The objective of this study was to define the conditions necessary for effective operation of the human erythrocyte [red blood cell (RBC)] acute toxicity assay.

PRIOR WORK

As reported in RFP-4036,[1] an acute toxicity assay using human erythrocytes was developed.

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METHOD

The method has been reported previously,[1] but briefly consists of the following steps:

Place 50 μℓ whole blood (diluted 1:1,000 in HEPES buffer) in a cuvette

Add 50 μℓ beryllium and mix

Incubate for 1 hour at 20 °C

Add 100 μℓ releasing agent

Place cuvette in photometer

Add 100 μℓ luciferin-luciferase

Record integrated light reading

The procedure was altered, one change at a time, and the effect of the change on the toxicity of beryllium to RBCs *in vitro,* as monitored by a decrease in adenosine triphosphate (ATP), was noted.

Following are the alterations made in our method and tested separately:

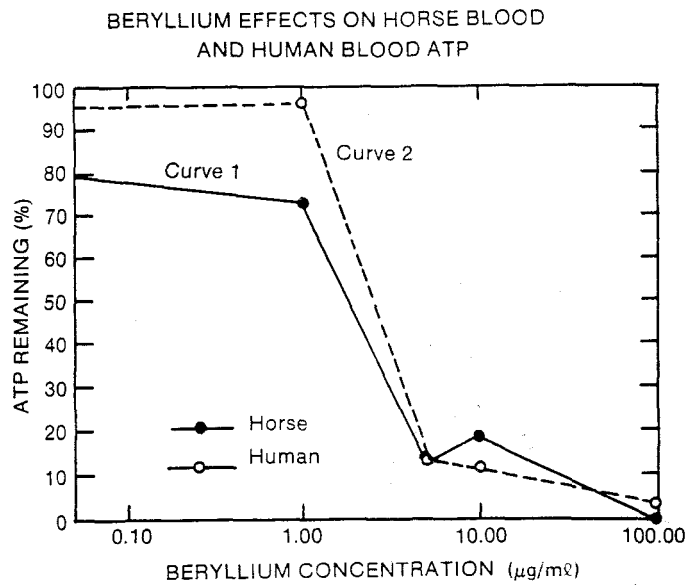
1. Horse blood was used instead of human blood as a source of ATP.
2. Human blood was diluted 1:1,000 in 0.9% sodium chloride (NaCl) or its homologous plasma instead of HEPES buffer.
3. The releasing agent was added before the toxicant (beryllium) instead of afterwards.
4. HEPES buffer was substituted for the releasing agent.
5. Purified ATP was substituted in place of human blood.

EXPERIMENTS AND RESULTS

Previously we have reported that ATP levels in blood from 10 RFP employees responded similarly to beryllium. These employees differed in sex, age, medication, and medical histories. To further check the constancy of this reaction, we tested the response using blood from a different species, horse blood. Figure 1, Curve 1, shows the response using horse blood is essentially the same as that using human blood (Figure 1, Curve 2).

When human blood was diluted in 0.9% NaCl or homologous plasma instead of HEPES buffer, the biphasic dose response curve was not produced. We found that the loss of ATP from blood diluted in 0.9% NaCl was approximately 25% after 1-hour incubation with 40 μg/mℓ Be (Figure 2, Curve 2) and only a 5% decrease under the same conditions when the cells were diluted in plasma (Figure 2, Curve 3). At 100 μg/mℓ Be, the cells diluted in 0.9% NaCl still retained 65% of their ATP content, but those in plasma dropped to 25%. This is in contrast to the blood diluted in HEPES (Figure 2, Curve 1) where nearly all the ATP was depleted.

FIGURE 1. Beryllium Effects on Horse Blood and Human Blood ATP. Horse blood (Curve 1) and human blood (Curve 2) diluted 1:1,000 in HEPES buffer and exposed to various concentrations of beryllium. Experiment was performed in duplicate and averaged.



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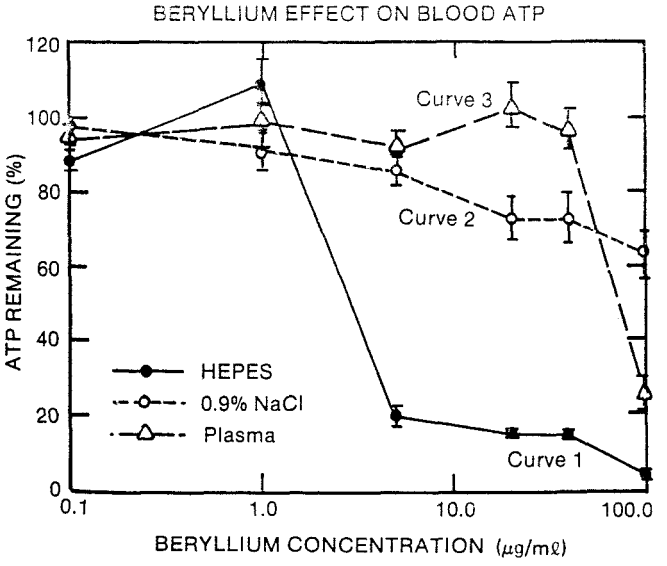


FIGURE 2. Beryllium Effect on Blood ATP. Beryllium concentration viability effect curves of red blood cells diluted 1:1,000 in HEPES, 0.9% NaCl, and plasma. The error bars represent the mean (± SEM) of three or four experiments performed in duplicate.

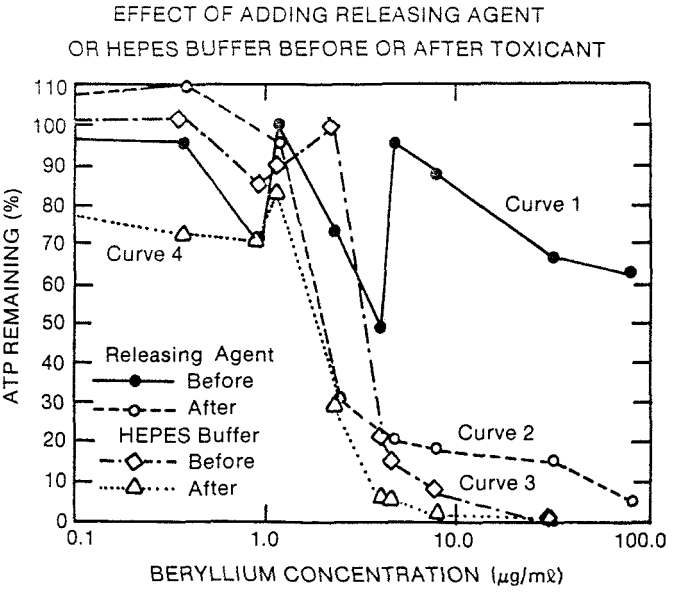
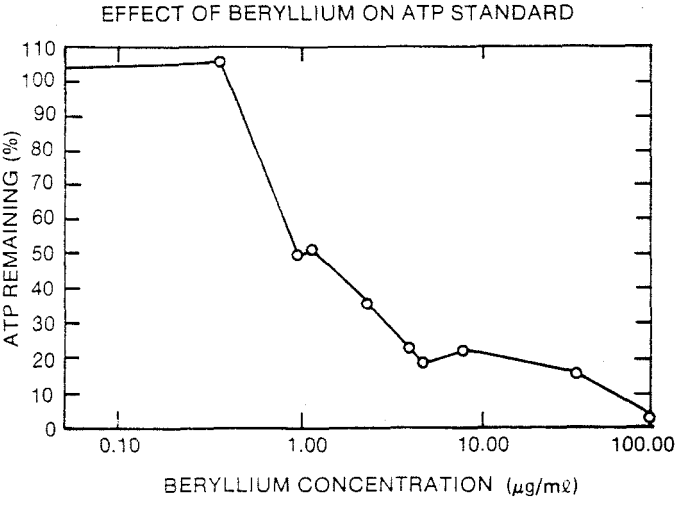


FIGURE 3. Effect of Adding Releasing Agent or HEPES Buffer Before or After Toxicant. Acute toxicity assay was performed by adding the releasing agent or HEPES buffer before or after the beryllium. Experiments were done in duplicate and averaged.

The role of the releasing agent was assumed to break down all membranes and release cellular ATP for measurement by the luciferin-luciferase enzyme system. The releasing agent also contained antiphosphatase to inhibit extra cellular phosphatases. When we added the releasing agent to the RBCs before the toxicant (beryllium), we saw an erratic effect (Figure 3, Curve 1), but when it was added after the toxicant had incubated for 1 hour with the RBCs, the biphasic toxic effect was seen (Figure 3, Curve 2). This was what we expected to find. However, when we substituted HEPES buffer for the releasing agent, we found that a toxic effect (i.e., decrease in ATP) was seen regardless of whether we added the buffer before or after the toxicant (Figure 3, Curves 3 and 4).

The biphasic dose response curve that we found when we treated RBCs with beryllium was thought to reflect the action of beryllium on the cell membrane and/or cytosol enzymes which would result in the decrease of ATP. To check this assumption, we substituted purified ATP diluted in HEPES buffer for the human RBCs. The results of the assay are shown in Figure 4. We were surprised

FIGURE 4. Effect of Beryllium on ATP Standard. Acute toxicity assay was performed using purified ATP (6.3 ng) instead of RBCs. Experiment was performed in duplicate and averaged.



to see a biphasic dose response similar to that obtained when RBCs are used. This reaction does not occur when RBCs or ATP is diluted in 0.9% NaCl (Figure 5).

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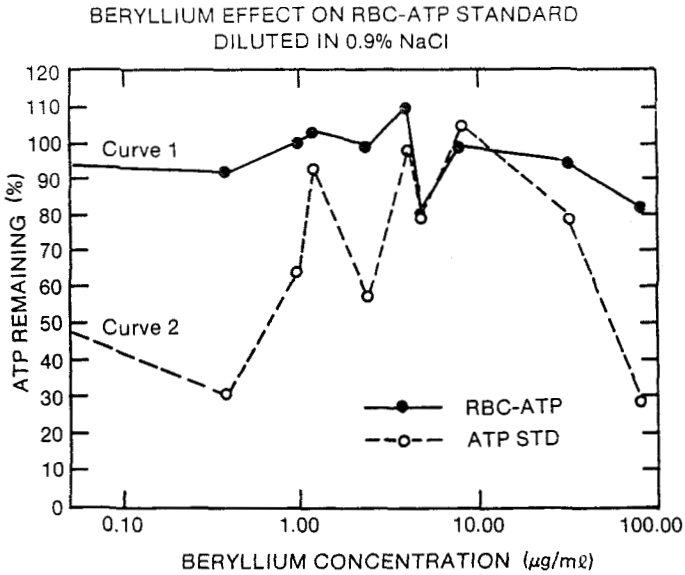

FIGURE 5. Beryllium Effect on RBC-ATP and ATP Standard Diluted in 0.9% NaCl. Acute toxicity assay was performed using either RBCs or purified ATP standard. The diluent for the RBCs or ATP was 0.9% NaCl instead of HEPES. The experiment was performed in duplicate and averaged.

Results of these experiments show that the dose response of blood is not dependent on species. However, the biphasic dose response curve is dependent on the buffer used to dilute the blood. Physiological buffers (0.9% NaCl and homologous plasma) seem to block the toxic effect of beryllium and this effect is only manifested by using a particular hypotonic buffer (Turner's proprietary HEPES).

The releasing agent appears to protect the ATP from the beryllium since less toxic effect is seen when it is added before the toxicant (beryllium). However, it appears that the releasing agent may not be necessary to release ATP since substitution of HEPES buffer resulted in the same toxic effect whether it was added before or after the toxicant. However, the releasing agent did appear to stabilize the reaction.

The most significant finding in these experiments was that purified ATP could be substituted for RBCs. This finding indicates that beryllium is acting directly on ATP. Previously we believed that beryllium was acting on the membrane and/or cytosol enzymes which would also reflect a decrease in ATP.

FUTURE WORK

This project is complete.

TESTING OF THE *IN VITRO* HUMAN ERYTHROCYTE ACUTE TOXICITY ASSAY

S. M. Clarke and C. W. Barrick

OBJECTIVE

The objective of this study was to test the assay's ability to detect toxic effects of nine substances used at Rocky Flats Plant (RFP) and to study the method reproducibility.

PRIOR WORK

This is a new project.

METHOD

To test reproducibility, the assay was run on four consecutive days using two different sources of human erythrocytes [red blood cells (RBCs)] and ten concentrations of toxicant (beryllium). The procedure used has been reported in the preceding progress report, RFP-4036.[1]

The ability to detect toxic effects was tested using nine different substances (aluminum, cadmium, copper, iron, mercury, phenol, lead, vanadium and beryllium). These substances are found at RFP and are used as toxicants in the acute toxicity assay. Two substances were tested each day for four consecutive days. Beryllium was tested each day as a control for variability.

EXPERIMEMTS AND RESULTS

Four assays done on four consective days using two different sources of blood were averaged and compared. Figure 6 shows the results of these experiments. Using nonparametric statistics, we

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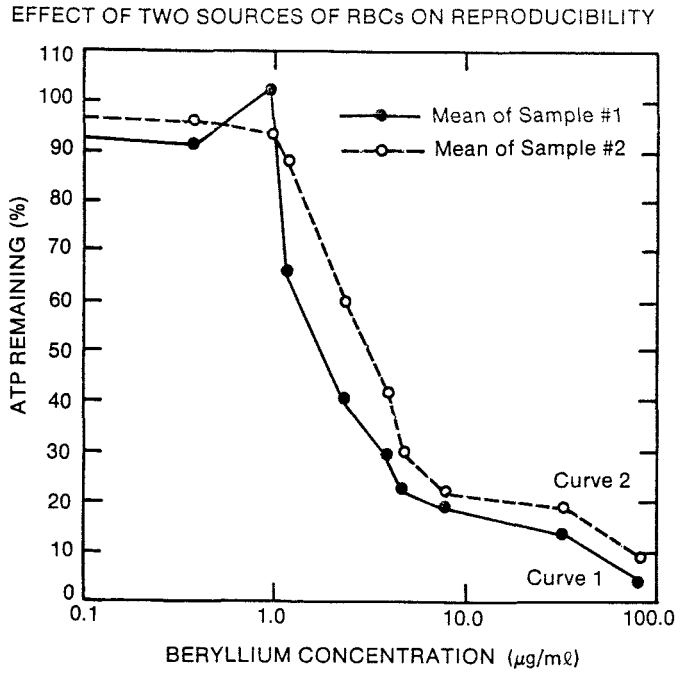


FIGURE 6. Effect of Two Sources of RBCs on Reproducibility. The human erythrocyte acute toxicity assay was run using two sources of blood against ten dilutions of beryllium on four consecutive days. Each point represents the average of triplicate samples.

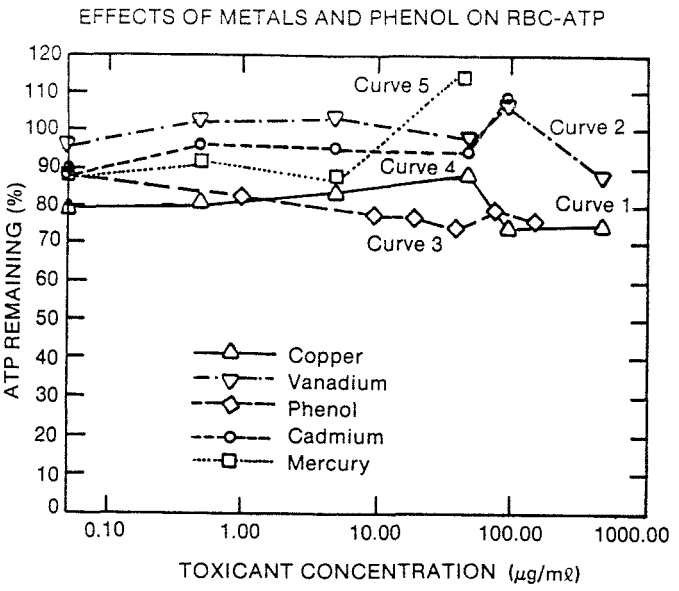
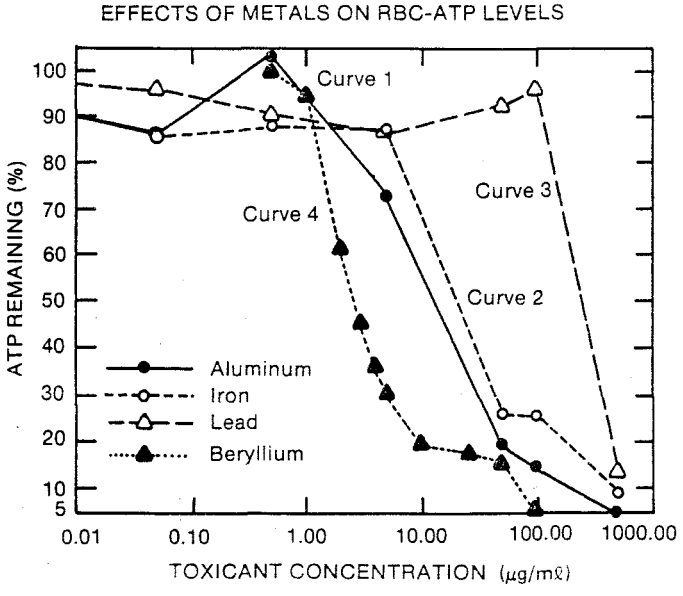


FIGURE 8. Effects of Metals and Phenol on RBC-ATP Levels. The human erythrocyte acute toxicity assay was run using four metals and phenol as the toxicants. Each point represents the average of triplicate samples.

FIGURE 7. Effects of Metals on RBC-ATP Levels. The human erythrocyte acute toxicity assay was run using four metals as the toxicants. Each point represents the average of triplicate samples.



found the two curves were not shown to be different at a 0.05 significant level.

Preliminary data on the nine substances tested for toxicity using this method showed that aluminum, iron, lead, and beryllium are toxic (Figure 7). The remaining five substances showed little to no toxicity to the RBC adenosine triphosphate (ATP) (Figure 8) as expressed by no decline in ATP content (% ATP remaining). An increase in ATP is seen at higher concentrations of some toxicants. This increase is due to undesired cross reaction with the internal controls rather than a stimulation of ATP production.

FUTURE WORK

These nine substances will be tested in the Microtox, Nematode, and SOS Chromotest assays and the results compared.

REFERENCE

1. L. C. Smith. *HS&E Application Technology Semiannual Progress Report July Through December 1985*, RFP-4036, Rockwell International, Rocky Flats Plant, Golden, CO, May 1, 1987.

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